3.0 MATERIALS & METHODS

3.1 Material

The leaves of *Allium tuberosum* (garlic chive), *Apium graveolens* (celery), *Ipomoea batatas* (sweet potato leaves), *Murraya koenigii* (curry leaves), *Psophocarpus tetragonolobus* (winged bean) and *Sauropus androgynus* (sweet leaves) were analysed for antioxidant potentials.The selected vegetables were obtained in New Malliga Stores, Brickfields. It was necessary to use materials which were free from disease, viral, bacterial or fungal infections to ensure accuracy of results.

3.2 Plant Preparation

The leaves of *Allium tuberosum*, *Apium graveolens* (L.), *Ipomoea batatas* (L.), *Murraya koenigii* (L.), *Psophocarpus tetragonolobus* and *Sauropus androgynus* were weighed before they were washed and dried in a hot oven at 40 °C for three days. The dried samples were weighed using (Mettler AJ100) before it was grounded into fine powder. The fine powder were kept sealed in a separate plastic bag.

3.3 Plant Extraction

Twenty gram (20 g) of each sample powder was used for the extraction process. Petroleum ether (200 ml) was added and the mixture was poured into a conical flask. The mouth of the conical flask was covered with aluminium foil and then placed in incubator shaker at 200 rpm for three days which was set at 34 ± 2 °C.

After three days, the mixture was filtered using 24 cm Whatman filter paper into round bottom flask. The concentrated extract (crude extract) was obtained using a rotary evaporator at 40-50 °C. The crude extracts were weighed and kept in a vial wrapped with aluminium foil to prevent oxidation. The residues from filtered powder were dried

and weighed to calculate the percentage of filtrate dissolved in the extract (Laetitia *et al.* 2008). The residues from filtered powder were extracted with the same procedure but using different solvents namely chloroform, methanol and finally water.

3.4 Antioxidant Assay

The petroleum benzene, chloroform, methanol and water extracts of *Allium tuberosum*, *Apium graveolens* (L.), *Ipomoea batatas* (L.), *Murraya koenigii* (L.), *Psophocarpus tetragonolobus* and *Sauropus androgynus* were evaluated for their antioxidant potentials using DPPH Radical Scavenging Assay, Reducing Power Assay, Metal Chelating Assay, Haemolysate Catalytic Assay and Lipid Hydroperoxide Assay.

3.4.1 DPPH Radical Scavenging Assay

This 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay evaluates the free radical scavenging activity of test extracts. The assay was carried out according to a method by Shimada *et al*. (1992) with slightly modification.

(i) Preparation of DPPH

A stock of 8 mg/ml of 1,1-diphenyl-2-picrylhydrazyl (DPPH) in methanol was prepared. The stock solution was kept in a flask wrapped with aluminium foil.

(ii) Ascorbic Acid as Positive References Standard

Ascorbic acid was used as the positive reference standard in the DPPH radical scavenging assay. A stock of ascorbic acid in methanol was prepared at a concentration of 400 μg/ml. The stock solution was kept in a flask wrapped with aluminium foil.

The reaction mixtures for ascorbic acid as reference standard were prepared as shown in Table 3.1. The final volume was 4500 µl. The reaction mixtures were allowed to react for 30 minutes at room temperature. The optical density was measured at 517 nm using spectrophotometer (Hitachi). Methanol was used as blank. The DPPH radical used alone without antioxidants served as the negative control.

Table 3.1: Reaction mixtures containing ascorbic acid, DPPH and methanol in the DPPH radical scavenging assay

Concentration of Ascorbic Acid (µg/ml)	Volume of Methanol, (µl)	Volume of Ascorbic Acid from stock, (µl)	Volume of DPPH, (µl)
0.0025	3990	10	500
0.0125	3950	50	500
0.0375	3850	150	500
0.125	3500	500	500
0.25	3000	1000	500
0.5	2000	2000	500
Control	4000	-	500

(iii) Screening for DPPH Radical Scavenging activity of Selected Plants Extracts

The screening of DPPH radical scavenging potential was performed to the selected plant extracts. The reaction mixture as shown in Table 3.2. The reaction mixtures were allowed to react for 30 minutes at room temperature. The optical density was measured at 520 nm using spectrophotometer (Hitachi). Methanol was used as blank. The DPPH radical used alone without any added extracts served as the negative control.

Table 3.2: Reaction mixtures containing crude extracts, DPPH and methanol in the DPPH radical scavenging assay

Concentration of Crude Extracts (µg/ml)	Volume of Methanol, (µl)	Volume of Crude Extract from stock, (µl)	Volume of DPPH, (µl)
0.0025	3990	10	500
0.0125	3950	50	500
0.0375	3850	150	500
0.125	3500	500	500
0.25	3000	1000	500
0.5	2000	2000	500
Control	4000	-	500

(iv) Determination of Percentage of Inhibition

The antioxidant activity of ascorbic acid and each extract was determined by the percentage of inhibition according to the following formula:

(Shimada *et al*., 1992)

$$\textbf{\% of inhibition} = \frac{\mathbf{OD_{control}} - \mathbf{OD_{sampel}}}{\mathbf{OD_{control}}} \times \mathbf{100}$$

Where:

$\mathbf{OD_{control}}$ = absorbance of the control

$\mathbf{OD_{sample}}$ = absorbance in the presence of the samples of crude extract

The control only contains methanol and DPPH without ascorbic acid/crude extract

3.4.2 Reducing Power Assay

This assay evaluates the ability of test extracts to reduce Fe^{3+} to Fe^{2+}. The reducing power assay was carried out according to a method by Oyaizu, (1986) with slightly modification.

(i) Reducing Power of Standard BHA

Butylated hydroxyanisole (BHA) was used as positive reference standard. BHA of different weights (1 mg, 0.5 mg, 0.25 mg, 0.125 mg and 0.0625 mg) was dissolved in 1.0 ml of methanol to which was added 2.5 ml of 0.2M phosphate buffer (pH 6.6) and 2.5 ml of a 1% (W/V) potassium ferricyanide (Sigma). The mixtures were incubated in a water bath at 50 °C for 20 minutes. Following this, 2.5 ml of a 10% (W/V) trichloroacetic acid solution (Sigma) was added and the mixture was then centrifuged at 1000 rpm for 10 minutes. A 2.5 ml aliquot of the upper layer was combined with 2.5 ml of distilled water and 0.5 ml of a 1% (W/V) solution of ferric chloride.

The colour changes were observed. The mixtures were then transferred into cuvettes. Optical density (OD) or absorbance of the reaction mixtures was read spectrophotometrically at 700 nm after 30 minutes of incubation. Increased absorbance of the reaction mixture indicates greater reducing power. Mean values from three independent test runs were calculated.

(ii) Reducing Power of Crude Extracts

Crude extracts at different concentrations (1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, and 0.0625 mg/ml) were tested for reducing power using the procedure described above (Table 3.3). All tests were carried out in triplicates and the readings were averaged. Mean values and standard deviation were calculated. Methanol was used as blank.

Table 3.3: Reaction mixtures containing crude extracts, phosphate buffer, potassium ferricyanide and ferric chloride

Concentration of crude extracts (mg/ml)	Phosphate Buffer 0.2M (mg/ml)	Potassium Ferricyanide (mg/ml)	Ferric Chloride (mg/ml)
0.0625	2.5	2.5	2.5
0.125	2.5	2.5	2.5
0.25	2.5	2.5	2.5
0.5	2.5	2.5	2.5
1.0	2.5	2.5	2.5

3.4.3 Metal Chelating Assay

Metal chelating assay evaluates the ability of test extracts to chelate ferrous ion and prevent the formation of ferrozine-Fe^{2+} complex. The chelating of ferrous ions by plant extracts was determined by the method of Dinis *et al.* (1994).

(i) Metal Chelating for EDTA Positive Reference Standard

EDTA (ethylenediaminetetraacetic acid) (Sigma) was used as positive reference standard in this assay. EDTA stock of 1 mg/ml was prepared by dissolving 0.01 g of EDTA in 4 ml deionized water. The pH was adjusted while stirring with NaOH solution until most of EDTA dissolved. Once the EDTA dissolved, deionized water was added to a final volume of 10 ml. 1 ml of various concentrations of EDTA at 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, 0.0625 mg/ml and control (without EDTA) were added to a solution of 2 mM $FeCl_2$ (0.05ml). The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml).

The mixture was shaken vigorously and left standing at room temperature for 10 minutes. The optical density (OD) of the mixture was then measured at 562 nm. Deionized water was used as blank. All tests were carried out in triplicates and the readings were averaged. Percentage of inhibition of ferrozine-Fe^{2+} complex each reading was calculated.

(ii) Metal Chelating for Crude Extracts

Crude extracts at different concentrations of 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, 0.0625 mg/ml and control (without crude extract) were tested for metal chelating activity using the procedure described above. Crude extract stock of 1 mg/ml was prepared by dissolving 0.01 g of crude extract in 10 ml methanol. All tests were carried out in triplicates and the readings were averaged. Percentage of inhibition of ferrozine-Fe^{2+} complex each reading was calculated.

(iii) Determination of Percentage of Inhibition

The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated using the formula given below: (Haro-Vicente *et al.*, 2006)

$$\textbf{\% of inhibition} = \frac{\mathbf{OD_{control}} - \mathbf{OD_{sampel}}}{\mathbf{OD_{control}}} \times \mathbf{100}$$

Where:

$\mathbf{OD_{control}}$ = absorbance of the control

$\mathbf{OD_{sample}}$ = absorbance in the presence of the samples of crude extract

The control only contains $FeCl_2$ and ferrozine without EDTA/crude extract .

3.4.4 Haemolysate Catalytic Activity

In haemolysate catalytic assay, the ability of *Allium tuberosum, Apium graveolens* (L.), *Ipomoea batatas* (L.), *Murraya koenigii* (L.), *Psophocarpus tetragonolobus,* and *Sauropus androgynus* to reduce hydrogen peroxide (H_2O_2) in synergism with haemolysate catalase was assessed.

(i) Preparation of haemolysate catalase

Blood sample of healthy individuals bought in University Hospital; Kuala Lumpur was drawn into an EDTA-vacutainer tube. The erythrocytes were hemolysed by a 10-fold dilution with sodium phosphate buffer pH 7.4 and kept on ice.

(ii) Preparation of stock solutions

Stock solutions of 1 M H_2O_2 and 20 μg/ml of crude extracts were prepared. 1 M H_2O_2 was prepared using distilled water and kept on ice. Stock solutions of crude extracts were prepared by dissolving the crude chloroform, methanol and water extracts of plant samples in sodium phosphate buffer pH 7.4.

(iii) Determination of the concentration of haemoglobin and total weight of haem in haemolysate

The concentration of haemoglobin in the haemolysate stock was calculated according to the Beer-Lambert's law (Ingle *et al*., 1988). The general Beer-Lambert law is written as:

$$A = \varepsilon bc$$

Where: A = absorbance

ε = wavelength-dependent molar absorptivity coefficient (M^{-1} cm^{-1})

b = path length

c = analyte concentration

The absorbance of the haemolysate stock was measured at 577 nm and the concentration of haemoglobin was calculated by using an extinction coefficient of 14.6 M^{-1} cm^{-1} for haemoglobin at 577 nm. The total weight of haem in the haemolysate stock was determined from the concentration of haemoglobin obtained by using the molecular weight of 616.49 amu for haem (Silberberg, 2006).

(iv) Activity measurement

The decomposition of H_2O_2 was measured using diode array spectrophotometer by determining the decrease in absorbance at 240 nm over the reaction time of 1.5 minutes. The activity of the haemolysate catalase alone in the reduction of H_2O_2 was used as the positive reference standard in this assay. Sodium azide (1 mM), the inhibitor of catalase, was used to confirm the reducing activity of catalase on H_2O_2.

The synergistic H_2O_2- reducing activities of the plant extracts were determined by measuring the decrease in H_2O_2 absorbance at 240 nm in the presence of haemolysate catalase. Reaction mixtures of plant crude extracts, haemolysate and H_2O_2 for the assay were prepared according to Table 3.4.

Table 3.4: Preparation of reaction mixture of plant crude extracts, haemolysate and H_2O_2 in haemolysate catalase assay

Concentration of crude extracts (µg/ml)	Volume of haemolysate (µl)	Volume of crude extracts from stock (µl)	Volume of H_2O_2 solution (µl)
50	3000	7.5	75
100	3000	15	75
200	3000	30	75
Standard	3000	-	75

(v) Determination of Percentage of Reduction of H_2O_2 by plant crude extracts

The percentage of reduction of H_2O_2 by the selected plant crude extracts was calculated using the formula given below:

$$\textbf{\% of inhibition} = \frac{\mathbf{OD_{control} - OD_{sampel}}}{\mathbf{OD_{control}}} \times \mathbf{100}$$

(Ilhami, 2005)

Where:

$\mathbf{OD_{control}}$ = absorbance of the control

$\mathbf{OD_{sample}}$ = absorbance in the presence of the samples of crude extract

The control is the haemolysate catalase alone without crude extract.

3.4.5 Lipid Hydroperoxide Assay

Lipid hydroperoxide assay is designed to measure lipid hydroperoxides in any sample type containing detectable levels. Quantification of lipid peroxidation is essential to assess the role of oxidative injury in pathophysiological disorders. Lipid peroxidation results in the formation of highly reactive and unstable hydroperoxides of both saturated and unsaturated lipids. The assay measures lipid hydroperoxides directly utilizing redox reactions with ferrous ion. Hydroperoxides are highly unstable and react readily with ferrous ions to produce ferric ions. The resulting ferric ions are detected using thiocyanate ion as the chromogen (Figure 3.1). The lipid hydroperoxide levels of plant extracts were carried out according to the protocol by Calbiochem® (Morrow & Roberts, 1997).

$$ROOH + Fe^{2+} \rightarrow RO^{\bullet} + Fe^{3+}$$

$$RO^{\bullet} + Fe^{2+} + H^{+} \rightarrow ROH + Fe^{3+} \qquad \mathbf{\lambda_{max} : 500\ nm}$$

$$Fe^{3+} + 5SCN^{-} \rightarrow Fe(SCN)_5^{2-} \qquad \mathbf{E : 16\,667\ M^{-1}cm^{-1}}$$

Figure 3.1: Reduction/Oxidation Reactions with Ferrous Ions

(i) Preparation of reagent

- Extract R

  100 mg Extract R was weighed into a tube and 15 ml methanol was added before vortexing for about 2 minutes. The methanol will become cloudy and most of the solid remains undissolved. The Extract R - saturated methanol should be used within 2 hours.

- Chromogen

  Chromogen is prepared by mixing equal volumes of FTS Reagent 1 and FTS Reagent 2 in a test tube and vortexed. Only enough chromogen for the number of samples being assayed was prepared, with each tube requiring 50 µl chromogen. Chromogen should be prepared immediately prior to use.

- Chloroform

  100 ml chloroform was deoxygenated by bubbling nitrogen through the solvent for at least 30 minutes. Part of the deoxygenated chloroform was cooled to 0 °C and stored on ice for extraction of the samples. Two volumes chloroform are required for the extraction of one volume sample.

- Methanol

  Approximately 100 ml methanol was deoxygenated by bubbling nitrogen through the solvent for at least 30 minutes.

- Chloroform:Methanol Mixture

  Two volumes of deoxygenated chloroform were mixed with one volume of deoxygenated methanol. The solvent mixture was ready for use in the assay. Approximately 1 ml chloroform: methanol mixture was needed for each assay tube.

- Lipid Hydroperoxide Standards

  The lipid hydroperoxide standard was stored at -20 °C and kept on ice during the experiment. 24 clean test tubes were prepared by labeling them A-H, in triplicate. The lipid hydroperoxide standard and chloroform: methanol mixture was mixed in each tube as described in the table below:

Table 3.5: Lipid Hydroperoxide Standard

TUBE	LIPID HYDROPEROXIDE STANDARD, (µl)	CHCL3-CH3OH, (µl)	FINAL CONCENTRATION LIPID HYDROPEROXIDES*, (nmol)
A	0	950	0
B	10	940	0.5
C	20	930	1.0
D	30	920	1.5
E	40	910	2.0
F	60	890	3.0
G	80	870	4.0
H	100	850	5.0

(ii) Preparation of sample

Plant materials should be homogenized in buffer containing no transition metal before use. Samples should be assayed immediately upon collection. If samples could not be assayed fresh, then the lipid hydroperoxides were extracted and then stored at -20 °C. The extracted lipid hydroperoxides are stable for at least one month at -20 °C.

Lipid hydroperoxides were extracted from the sample with chloroform before performing the assay. This extraction step ensures the elimination of nearly all interfering substances from a sample. A known volume of sample (e.g., 500 µl plant extract) was aliquoted into a glass test tube and an equal volume of Extract R-saturated methanol was added to each tube and vortexed. 1 ml of cold deoxygenated chloroform was added to each tube and mixed thoroughly by vortexing. The mixture was centrifuged at 5792 rpm for 5 minutes at 0 °C. The bottom chloroform layer was collected by carefully inserting a Pasteur pipe along the side of the test tube. The chloroform layer was transferred to another test tube and stored on ice.

(iii) Detailed Protocol

500 µl chloroform extract of each sample was added to appropriately labeled glass test tubes. Any transfer of water from the extract was avoided. 450 µl of chloroform: methanol mixture was added to each sample tube. 50 µl of freshly prepared Chromogen was added to each tube containing lipid hydroperoxide standards and samples and the mixture was vortexed. The tubes were closed tightly with polypropylene caps. The mixture was incubated at room temperature for 5 minutes. The absorbance of each tube was measured at 500 nm using either a glass or quartz 1 ml cuvettes. The chloroform: methanol mixture was used for the blank if the spectrophotometer requires it. The colour of the chromogen is stable for 5 hours. If the tubes were not tightly capped, evaporation of solvent will result in a change in the absorbance.

(iv) Lipid hydroperoxide level measurement

The average absorbance for each lipid hydroperoxide standard and sample was calculated. The average absorbance of lipid hydroperoxide standard A was substracted from itself and all other lipid hydroperoxide standards and samples. The corrected

absorbance of lipid hydroperoxide standards was plotted as a function of final hydroperoxide value. The hydroperoxide values of the sample tubes (HPST) was calculated using the equation obtained from the linear regression of the standard curve substituting corrected absorbance values for each sample.

HPST (nmol) = (sample absorbance-y-intercept)/slope

The concentration of hydroperoxide in the original sample was calculated as shown below: (Porter *et al.*, 1995)

Volume of extract used for the assay = VE(ml)

Volume of the original sample used for extraction = SV(ml)

$$\textbf{Hydroperoxide concentration in sample (µM)} = \frac{\textbf{HPST}}{\textbf{VE}} \times \frac{\textbf{1ml}}{\textbf{SV}}$$

Assay Range: 0.25-5 nmol hydroperoxide per assay tube.

3.4.6 Separation by Thin Layer Chromatography

The petroleum benzene, chloroform, methanol and water extracts of *Allium tuberosum*, *Apium graveolens* (L.), *Ipomoea batatas* (L.), *Murraya koenigii* (L.), *Psophocarpus tetragonolobus* and *Sauropus androgynus* were evaluated using the thin layer chromatography technique. Thin layer chromatography technique is the most rapid and easily applied method to separate the organic compounds in a sample. A line was drawn on the TLC sheet about 1 cm from the top and bottom using pencil. The elution of each extracts was spotted onto the TLC sheet about 3 mm in diameter using capillary tube. The spotting solvent was evaporated for a few seconds and then the TLC sheet was placed in the TLC developing chamber filled with 100% ethyl acetate with the sample spots above the solvent level. Due to toxicity, cost, and flammability concerns, the common solvents 100% ethyl acetate (an ester) was chosen. After developing, the TLC sheet was then removed from the developing tank and allowed to dry. The spots on TLC

plate were visualized by using UV light, Iodine vapor visualization, acid sulphuric visualization and dragendorff reagent (Harder & Qian, 1999; Yin *et al*., 2008). The distance travelled by each component of the spot on the TLC plate expressed as a rate of retardation factor R_f. The R_f value calculated by dividing the distance travelled by a component with the distance between the origin and solvent front (http://orgchem.colorado.edu/hndbksupport/TLC/TLCrf.html).

$$R_f = \frac{\text{Distance travelled by the compounds}}{\text{Distance travel by solvent front}}$$

(i) UV light Visualisation

The TLC sheets that have been developed and dried were then observed using ultra visible light. There are two different wavelength used to visualize the spots. The short wavelength (254 nm) of UV light was used to detect substances that quench fluorescence. The appearance of dark spots or zones against a yellow green background indicates the presence of compounds with double conjugated and presence of fluorescence spots were recorded. Then, UV light at a longer wavelength (365 nm) was used for the detection of substance including most compounds with aromatic rings and conjugated double bonds and some unsaturared compounds that fluorescence in long wave UV light and the fluorescence spots were recorded (Luo *et al*., 2009).

(ii) Iodine Vapour Visualisation

Iodine vapour is known as unspecific universal reagent for many organic compounds. The chamber was charged with some crystals of iodine until the air in the chamber was saturated with iodine vapour. Developed and dried TLC plate was placed in the chamber with saturated iodine vapour. The spots turned into tan-brown colour that indicating the presence of organic compounds (Harder & Qian, 1999).

(iii) Acid Sulphuric Visualisation

Acid sulphuric with the volume of 10.8 ml was diluted in 200 ml distilled water. Then, the prepared acid sulphuric was sprayed onto the TLC plate and heated at 100 °C in an oven for five to ten minutes to detect the presence of spots. This reagent was used to detect the presence of terpenoids (Harder & Qian, 1999).

(iv) Dragendorff's Reagent Visualisation

Dragendorff's visualization was to detect the presence of nitrogen compounds, alkaloids, antiarrhythmic drugs and surfactants in the sample. Dragendorff's reagent was added to 5 µL of each collected extracts with the ratio of 1:1. Orange colour deposition indicates the presence of alkaloid compounds (Yin *et al*., 2008).